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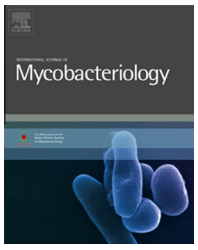

Short Communication

Use of rapid molecular test for multidrug-resistant tuberculosis detection among relapse cases in Cote d'Ivoire

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ABSTRACT

Tuberculosis is explicitly recognized as a major global public health problem. In Côte d'Ivoire, relapse cases represent 66.5% of patients eligible for retreatment according to the National Tuberculosis Control Program. This study objective was to detect multidrug-resistance tuberculosis among relapse cases. Patients were recruited in tuberculosis centers in routine. A standardized questioning was administrated. Two sputum samples were collected and transported at Institut Pasteur. Sputum samples were decontaminated by NALC method. The DNA extraction was realized with 500 µl of decontaminated sputum sample with smear-positive. MTBDRplus assay version 2.0 was performed according to the manufacturer's instruction. An internal quality control program with positive and negative controls was implemented for interpretation of results. In total 146 relapse cases with smear positive were studied. Out of selected patients, 130 had received the 2RHZE/4RH regimen and 16, the 2RHZES/1RHZE/5HRE. In group of relapse cases previously treated with 2RHZE/4RH regimen, 40 (31.3%, IC95%: [0.23; 0.39]) had punctual mutations at codon 526 in *rpoB* gene. Although, in patients under treated with 2RHZES/1RHZE/5HRE, a mutation in *rpoB* gene was identified in 12 of 16 sputum samples. Thirteen mutations conferring a resistance to Isoniazid were observed of which 9 in *katG* gene and 4 in *katG* and promoter region of inhA gene. The comparison (Chi-square with Yates correction) of resistance rates to Rifampin estimated showed a statistically significant difference.

Conclusion: Use of a rapid method to detect drug-resistance in recurrent TB cases has permitted to identify patients eligible for first-line drugs or not.

Introduction

Tuberculosis (TB) is a disease that affects the whole society and its drug-resistant form adds another obstacle to its control. Rapid diagnosis of TB and multidrug-resistant (resistance to at least Rifampin and Isoniazid) TB (MDR-TB) is an important challenge to ensure a quick and adequate course of TB therapy is initiated to limit the dissemination of

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multi-resistant strains [1]. Rifampin and Isoniazid are the cornerstones of first-line drugs used for the treatment of TB.

With DNA fingerprinting, the cases of recurrent TB can be categorized as relapse cases due to the original infecting strain or reinfection with a new strain of *Mycobacterium tuberculosis* [2]. Considering that recurrent TB cases are also the previously treated patients for TB, they are consequently a group with high risk for MDR-TB [3].

At the operational level, the recurrent TB cases are often identified as relapse cases. In fact, a relapse case is a patient previously declared cured or treatment completed and subsequently diagnosed with a new episode of TB bacteriologically confirmed (sputum smear or culture) [4]. The emergence of MDR-TB associated with relapse may be considered as an additional challenge in TB control [5,6], notably in developing countries. The prevalence of MDR-TB has been estimated to be low in sub-Saharan Africa, where surveillance of drug resistance is limited [1]. In Côte d'Ivoire, the prevalence of MDR-TB cases among new cases with a positive smear decreased from 5.3% to 2.5%, respectively from 1995 to 2006 [6].

These last 10 years, relapse cases represented 66.5% of retreatment cases notified by the National Tuberculosis Control Program. Relapse cases may be to a certain extent assimilated with new TB cases. Also, there is limited data available for the principal two first-line drugs in Côte d'Ivoire concerning relapse cases.

New diagnostic tools for TB were developed and validated, particularly molecular methods [7,8]. Molecular methods are highly efficient for Rifampin resistance detection and are well adapted to search for the most relevant Isoniazid resistance. Some methods require minimal infrastructure and equipment [9].

The study objective was to detect resistance to Rifampin and Isoniazid among relapse cases diagnosed in TB centers in Côte d'Ivoire.

Patients and methods

Study sites

Patients were recruited from the Pneumology service of CHU de Cocody and also referrals from regional centers for TB in Côte d'Ivoire. In a sanitary pyramid, the pneumology service is at the central level, and the regional centers are at the intermediate level. A standard questionnaire was administered by trained medical staff. Symptomatic patients were included consecutively based on their history [4]. Data collected included age, gender, previous anti-TB treatment, episodes of TB and results of their smears for AFB detection after Ziehl-Neelsen staining.

Samples collection and transport

For each patient enrolled, two sputum samples (spot, early morning) were collected and put in individual bags. Samples collected were transported at 4 °C in the icebox from the study site to the National Reference Laboratory for Tuberculosis. This laboratory is integrated in the Mycobacteria Unit of Institut Pasteur de Côte d'Ivoire.

Resistance detection

DNA extraction

Sputum samples were processed using the US Centers for Disease Control and Prevention (US CDC) recommended method of N-acetyl-L-Cysteine 4% NaOH-2.9% Citrate. Five milliliters of sputum were transferred in 50 ml of plastic centrifuge tube. Five milliliters of NALC solution were added to the sputum. The preparation was mixed by vortexing for 1 min and incubated at room temperature for 15 min. In the centrifuge tube, 35 ml of sterile phosphate buffer pH 6.8 were added and centrifuged at 3.000*g* for 20 min. Supernatant was carefully eliminated. Pellet was re-suspended with 2 ml of sterile phosphate buffer; 200 μl of re-suspended pellet were used to perform a smear which was stained using the Ziehl-Neelsen method.

The "GenoLyse®" kit was used for bacterial DNA extraction; 500 microliters of sediment were transferred in an eppendorf tube of 1500 μl. The suspension was centrifuged at 10.000*g* in an aerosol-tight rotor for 15 min. The supernatant was discarded; 100 μl of lysis buffer were added to the sediments. The bacterial preparation was homogenized by vortexing. The bacterial suspension was inactivated at 95 °C for 5 min; 100 μl of neutralization buffer were added to the preparation. The inactivated suspension was centrifuged at 13.000*g* for 5 min. The DNA contained in the supernatant was transferred to a fresh tube. A negative control was included in each run of sputum sample decontaminated for DNA extraction.

Amplification of DNA extracted from sputum samples

With sputum containing AFB, Genotype MTBDR assay version 2.0 (Hain Lifescience, Nehren, Germany) was performed as recommended by the manufacturer. The amplification mixture contained 35 μl of primer-nucleotide Mix B, 10 μl of Mix A (5 μl 10× PCR buffer, 2 μl of MgCl2, 3 μl of molecular water, 1 unit of thermostable *Taq* DNA polymerase) and 5 μl of extracted chromosomal DNA solution.

Amplification parameters used were: 15 min of denaturation at 95 °C, followed by 20 cycles of 30 s at 95 °C and 2 min at 65 °C, followed by 30 additional cycles of 25 s at 95 °C, 40 s at 53 °C, and 40 s at 70 °C, ending with a final extension step of 8 min at 70 °C (1 cycle).

Prior to hybridization, a migration of 5 μl of each PCR product was performed during 30 min at 100 V in 1% agarose gel stained with ethidium bromide. DNA amplified was revealed with a UV-transilluminator lamp (wave's length 365 nm).

Hybridization

Hybridization and detection were performed with a TwinCubator semi-automated washing and shaking device according to the manufacturer's instructions and using the reagents provided with the kit. Twenty microliters of denaturation solution was mixed with 20 μl of amplified sample. The mixed solution was incubated at room temperature for 5 min. One milliliter of pre-warmed hybridization buffer was added before the membrane strips were placed and shaken in the hybridization solution for 30 min at 45 °C. After two washing steps, a colorimetric detection of the hybridized amplicons

was obtained by the addition of the streptavidin alkaline phosphatase conjugate.

An internal quality control program with positive and negative controls was implemented during the study. An interpretable MTBDR*plus* assay was defined as a test strip with all control markers positive, including results of the markers for positive control (H37Rv strain), negative control for DNA extraction and for mix preparation.

Statistical tests

Data were entered in the MS Excel 8.0 and analyzed using Epi-info 6.04 (CDC, Atlanta). The rate of resistance to Rifampin and Isoniazid in each category of patients recruited with 95% Confidence Interval (CI) was estimated. The rates obtained were compared using Chi-square testing.

Results

A total of 146 relapse cases with positive smear were selected. After the analysis of PCR products with a UV-transilluminator lamp, all were definitively enrolled into the study. Among these 146 patients, 130 had received the 2RHZE/4RH regimen and 16 the 2RHZES/1RHZE/5HRE regimen. The internal control quality results were concordant with expected results. Indeed, the negative control included in each serial of DNA extraction was negative for detection of DNA amplified. The negative and positive controls of each testing run have been also respectively positive and negative (Fig. 1).

In the group of relapse cases previously treated with 2RHZE/4RH regimen, analysis of the sputum samples revealed 40 (31.3%, IC95%: [0.23; 0.39]) punctual mutations in *rpoB* gene. Most of them occurred at codon 526. Two cases of double mutation in this gene were observed in 2 sputum samples.

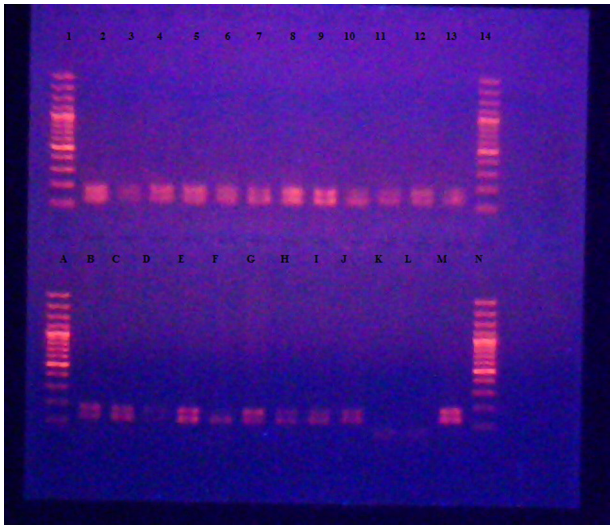

Fig. 1 – Revelation of PCR products after migration. Legend: lines 1, 14, A and N: molecular weight marker (100 pb). Lines from 2 to 13 and from B to J amplified DNA of sputum samples. Line K: negative control for mix preparation. Line L: negative control for DNA extraction. Line M: positive control (H37rv).

A S315T mutation (*katG* gene) was observed in 29 sputum samples of recruited patients. A mutation in *katG* gene associated with a mutation in the promoter region was described in the sputum of 13 patients. Among the 130 sputum samples analyzed, 39 (30.5%) had a simultaneous mutation in *rpoB* and *katG* genes (Table 1).

In the second group of patients (treated with 2RHZES/1RHZE/5HRE), a mutation in the *rpoB* gene was identified in 12 of the 16 sputum samples analyzed. Thirteen mutations conferring a resistance to Isoniazid were observed, of which 9 in the *katG* gene and 4 in the *katG* and promoter region of the *inhA* gene. Among 15 sputum samples analyzed and validated, 10 (66.7%) had a mutation in the *rpoB* gene associated with a mutation in one of the two genes (*katG* gene, *inhA* promoter region). Among these 15 sputum samples, 2 had no mutation in the genes explored (Table 1).

The comparison (Chi-square testing with Yates correction) of resistance rates to Rifampin estimated from sputum samples in the two groups of relapse cases showed a statistically significant difference (Table 2).

Discussion

At the operational level of the sanitary pyramid, a patient treated for TB and declared “cured” or “treatment completed” that presents with a new episode of TB confirmed bacteriologically is considered a relapse case [4]. From the bacteriological point of view, this new episode of TB can be due to an infection with the strain of the first episode or by re-infection with a new strain of *M. tuberculosis* Complex.

Relapse cases or recurrence TB cases [10] represent a group of patients who have a risk for drug resistances and MDR-TB [11]. An efficient tool is required for identification of MDR-TB cases. In most countries, effective control of drug-resistant TB requires the rapid detection of drug strains followed by prompt implementation of an adequate anti-TB therapy based on laboratory findings. This is a challenge for National Tuberculosis Control Programs notably in developing countries. Although performances of the molecular assay have not been estimated in this study, the method used has been developed and evaluated for use with *M. tuberculosis* cultures and smear-positive specimens [12,13]. In different TB laboratories, evaluations of this molecular method showed differences in terms of susceptibility and specificity [7,12,13]. In Africa, some studies were realized. Results of evaluation directly from sputum samples in Uganda [14] revealed a sensitivity of 100% and 96.1% of specificity for detection of Rifampin resistance. For Isoniazid resistance, sensitivity and specificity were respectively 80.8% and 100.0%.

Despite the precautions taken, two cases of hybridization were uninterpretable (Table 2). Analysis of laboratory data (date of DNA extraction, DNA migration and revelation, line probe assay results obtained. . .), notably for the double mutation obtained at the 526 codon combined with internal quality control results obtained for each run of DNA extraction, mix preparation, and hybridization have shown that these results were due to contamination of DNA contained in the tube during the process of DNA extraction [15]. Error occurred during transfer of DNA into a fresh tube. This kind of contamination

Table 1 – Results of Rifampin and Isoniazid susceptibility detection in sputum samples.

Mutations	Relapse after regimen	
	2RHZE/4RH *n* = 130	RHZES/1RHZE/5HRE *n* = 16
rpoB		
*rpoB*Mut1	10	2
*rpoB*Mut2A	11	1
*rpoB*Mut2B	9	4
*rpoB*Mut3	10	4
rpoB Mut1 + *rpoB*Mut2B	1	–
rpoB Mut2A + *rpoB*Mut2B	1[a]	1[a]
rpoB wild type	88	4
katG		
katG Mut1	29	9
katG wild type	88	3
inhA promoter region		
inhA Mut1	–	–
inhA Mut2	–	–
inhA Mut3A	–	–
inhA Mut3B	–	–
inhA wild type	117	12
Associated mutations		
katG Mut1 + *inhA* Mut3A	8	4
katG Mut1 + *inhA* Mut3B	5	–

[a] Mutations concerning the same codon.

Table 2 – Comparison of mutation rate observed in the categories of relapses for *rpoB gene*.

Mutation in *rpoB*	2RHZE/4RH *n* = 128	2RHZES/1RHZE/5HRE *n* = 15
Presence of one mutation	40	11
Absence of mutation	88	4

Chi-square testing with Yates correction: 8.61 *p* value = 0.0003.

may be reduced by reducing the workload of technicians. The method used seems most adapted for laboratories at the central level.

A previous treatment for TB is a risk factor associated with drug-resistant *Mycobacterium* [11]. Indeed, some studies showed a high rate of MDR-TB cases estimated at 12.1% and 9.2% in patients previously treated in Uganda and Gujarat (India), respectively [3,16].

In this study, all MDR-TB cases detected were previously treated with only first-line drugs. For relapse cases who had received the 2RHZE/4RH regimen, 39 (30.5%) among the 128 patients with validated results were MDR-TB cases.

In Vietnam, Quy et al. have shown that among relapse cases, none had primary resistance. The MDR-TB cases estimated to 8% in their study were acquired. Consequently, these cases were probably due to infection with a new strain. [17].

The rate of MDR-TB cases observed in relapse cases after the 2RHZES/1RHZE/5HRE regimen was twice as important as the one of the group treated with 2RHZE/4RH. The difference observed between these two groups may be explained by antecedents of treatment of the patients who received the retreatment regimen [11].

The MDR-TB rates observed are high in recurrent TB cases. In this setting, Directly Observed Therapy (DOT) must be improved for reducing the dissemination of strains as one of the principal sources of re-infection in recurrent TB cases [18]. These efforts must also be sustained by an improvement in the social and economic context (housing, well-being, public transport...).

In this study, the molecular method used has permitted the detection of one case of infection with two strains. Indeed, the eight probes which detect a mutation in the *rpoB* gene were hybridized and revealed (Table 2). It is not clearly established that this observation is due to mixed infection. In the endemic context of TB, mixed infection is not exceptional, but it must be well documented [19], notably in previous patients treated for TB.

Conclusion

Use of a rapid method to detect drug-resistance in recurrent TB cases has allowed for an estimate of multidrug-resistant TB and clearly identifies patients eligible for first-line drugs or not.

Conflict of interest

None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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REFERENCES

[1] The World Health Organization (WHO)–International Union Against Tuberculosis and Lung Disease (IUATLD), "Global project on antituberculosis drug resistance surveillance. Anti-tuberculosis drug resistance in the world": fourth global report. WHO/HTM/TB/2008.394.

[2] R.M. Jasmer, L. Bozeman, K. Schwartzman, M.D. Cave, J.J. Saukkonen, B. Metchock, et al, The tuberculosis trials consortium. Recurrent tuberculosis in the United States and Canada: relapse or reinfection, Am. J. Respir. Crit. Care Med. 170 (2004) 1360–1366.

[3] A.R. Shah, S.K. Agarwal, K.V. Shah, Study of drug resistance in previously treated tuberculosis patients in Gujarat, India, Int. J. Tuberc. Lung Dis. 6 (2002) 1098–1101.

[4] World Health Organization, International Union against Tuberculosis and Lung Disease, Royal Netherlands Tuberculosis Association, Revised international definitions in tuberculosis control, Int. J. Tuberc. Lung Dis. 5 (2001) 213–215.

[5] C. Dye, M.A. Espinal, C.J. Watt, C. Mbiaga, B.G. Williams, Worldwide incidence of multidrug-resistant tuberculosis, J. Infect. Dis. 185 (2002) 1197–1202.

[6] K. N'guessan, I. Nahoua, M. San Koffi, J. Kouakou, M. Dosso, Primary resistance to anti-tuberculosis drugs: trends in Côte d'Ivoire from to 2006, Med. Mal. Infect. 38 (2008) (1996) 231–232.

[7] F. Brossier, N. Veziris, C. Truffot-Pernot, V. Jarlier, W. Sougakoff, Performance of the genotype MTBDR line probe assay for detection of resistance to Rifampin and Isoniazid in strains of *Mycobacterium tuberculsosis* with low and high level resistance, J. Clin. Microbiol. 44 (2006) 3659–3664.

[8] M. Bates, J. O'Grady, M. Maeurer, J. Tembo, L. Chilukutu, C. Chabala, et al, Assessment of the Xpert MTB/RIF assay for diagnosis of tuberculosis with gastric lavage aspirates in children in sub-Saharan Africa: a prospective descriptive study, Lancet Infect. Dis. 13 (2013) 36–42.

[9] P.P. Banada, S.K. Sivasubramani, R. Blakemore, C. Boehme, M.D. Perkins, K. Fennelly, et al, Containment of bioaerosol infection risk by the Xpert MTB/RIF assay and its applicability to point-of-care settings, J. Clin. Microbiol. 48 (2010) 3551–3557.

[10] A.C. Crampin, J.N. Mwaungulu, F.D. Mwaungulu, D.T. Mwafulirwa, K. Munthali, S. Floyd, et al, Recurrent TB: relapse or reinfection? The effect of HIV in a general population cohort in Malawi, AIDS 24 (2010) 417–426.

[11] J.A. Caminero, Multidrug-resistant tuberculosis: epidemiology, risk factors and case finding, Int. J. Tuberc. Lung Dis. 14 (2010) 382–390.

[12] D. Hillemann, S. Rüsch-Gerdes, E. Richter, Evaluation of the genotype MTBDRplus assay for Rifampin and Isoniazid susceptibility testing of *Mycobacterium tuberculosis* strains and clinical specimens, J. Clin. Microbiol. 45 (2007) 4459–4463.

[13] A. Somoskovi, J. Dormandy, D. Mitsani, J. Rivenburg, M. Salfinger, Use of smear-positive samples to assess the PCR-based genotype MTBDR assay for rapid, direct detection of the *Mycobacterium tuberculosis* complex as well as its resistance to Isoniazid and Rifampin, J. Clin. Microbiol. 44 (2006) 4459–4463.

[14] H. Albert, F. Bwanga, S. Mukkada, B. Nyesiga, J.P. Ademun, G. Lukyamuzi, et al, Rapid screening of MDR-TB using molecular line probe assay is feasible in Uganda, BMC Infect. Dis. 10 (2010) 41, http://dx.doi.org/10.1186/1471-2334-10-41.

[15] M. Barnard, L. Parsons, P. Miotto, Cirillo, K. Feldmann, Gutierrez, et al, Molecular Detection of Drug-Resistant Tuberculosis by Line Probe Assay: Laboratory Manual for Resource-Limited Settings, Foundation for innovative New Diagnostics, Geneva, 2012.

[16] D. Lukoye, F. Adatu, K. Musisi, G.W. Kasule, W. Were, R. Odeke, et al, Anti-tuberculosis drug resistance among new and previously treated sputum smear-positive tuberculosis patients in Uganda: results of the first national survey, PLoS ONE 8 (2013) e70763.

[17] H.T.W. Quy, N.T.N. Lan, M.W. Borgdorff, J. Grosset, P.D. Linh, L.B. Tung, et al, Drug resistance among failure and relapse cases of tuberculosis: is the standard re-treatment regimen adequate?, Int J. Tuberc. Lung Dis. 7 (2003) 631–636.

[18] J.R. Andrews, N.R. Gandhi, P. Moodley, N.S. Shah, L. Bohlken, A.P. Moll, et al, Exogenous reinfection as a cause of multidrug-resistant and extensively drug-resistant tuberculosis in rural South Africa, J. Infect. Dis. 2008 (198) (2008) 1582–1589.

[19] K. Mallard, R. McNerney, A.C. Crampin, R. Houben, R. Ndlovu, L. Munthali, et al, Molecular detection of mixed infections of *Mycobacterium tuberculosis* strains in sputum samples from patients in Karonga district, Malawi, J. Clin. Microbiol. 48 (2010) 4512–4518.